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Expression of Type IV Collagen and Its Degrading Enzymes in Squamous Cell Carcinoma of Lung

Hideyuki Nakagawa and Soroku Yagihashi[1]

First Department of Pathology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036

We examined the *in situ* distribution of basement membrane collagen (Col IV), matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) by immunohistochemistry and their mRNA levels by Northern blot analysis in 14 cases of squamous cell carcinoma of the lung. Elevated mRNA levels of MMP-2 and Col IV were demonstrated in all the cases examined and were associated with *in situ* disruption of basement membranes around the tumor nests. In contrast, TIMP-1 mRNA levels were not altered. MMP-2, MMP-9 and TIMP-1 were localized in tumor cells, stromal fibroblasts and endothelial cells. There were no significant correlations between these parameters and clinical staging. The results suggest that the degrading enzymes of basement membrane collagen play an important role in the invasion and metastasis of human squamous cell carcinoma of the lung.

Key words: Lung cancer — Squamous cell carcinoma — Type IV collagen — Matrix metalloproteinase — Tissue inhibitor of metalloproteinase

Destruction of basement membrane collagen is the first step in the invasion and metastasis of cancer. Enhanced expression of metalloproteinases (MMPs) was reported in human cancers of colon,[1] ovary,[2] and esophagus.[3,4] Garbisa *et al.*[5] found a significant increase in MMP-2 levels in the sera from lung cancer patients and a correlation between clinical staging and serum MMP-2 levels. It is not known, however, whether the expression of type IV collagen (Col IV) and its degrading enzymes are altered in pulmonary squamous cell carcinoma (SCC).

In this study, we evaluated the expression of Col IV, MMP-2, MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) of human SCC of the lung by immunohistochemistry and Northern blot analysis, and compared these data with clinicopathological features.

MATERIALS AND METHODS

Fourteen samples of surgically removed primary lung SCC were used in this study. A half of each specimen was immediately frozen at −80°C and stored for Northern blot analysis. The other half was fixed with 10% buffered formalin and embedded in paraffin. Clinical staging and histological differentiation were determined based on the general rules for clinical and pathological recording of lung cancer in Japan.[6] The clinicopathological data for these patients are summarized in Table I. Specimens of normal lung tissues without inflammatory changes adjacent to the lung tumor were pooled and served as normal controls.

For microscopic observations, serial sections of 4 μm thickness from paraffin-embedded tissues were stained with hematoxylin and eosin, and Victoria blue. The degree of lymphatic invasion was graded on the Victoria blue-stained sections; i.e., ly_0, no invasion; ly_1, invasion of 1 or 2 vessels; ly_2, 3 to 4 vessels; ly_3, invasion of more than 5 vessels. The degree of blood vessel invasion was also graded as v_0, v_1, v_2 and v_3 in a similar manner to that used for lymphatic invasion.

Immunohistochemical staining by the streptavidin-biotin method (Histofine SAB kit, Nichirei, Tokyo) was performed on the serial sections using the following antibodies; i.e., anti-human Col IV (Dako, Glostrup, Denmark), anti-human 72 kDa collagenase (Molecular Oncology, Maryland, USA), and anti-human TIMP-1 (Fujiyakuhin, Takaoka). MMP-2 and TIMP-1 immunoreactivities were evaluated as negative (−) when no positive cells were found, weakly positive (±) when positive tumor cells were less than 10%, positive (+) when 10 to 50% of tumor cells were positive, and strongly positive (++) when over 50% were positive.

For the measurement of mRNA levels, total RNA from frozen tissues was extracted with Isogen (Nippon Gene, Osaka). Twenty μg of total RNA samples electrophoresed on 1% agarose gel was transferred onto nylon membrane (NEN, Boston, USA) and hybridized with ^{32}P-labeled cDNA probes (10^6 cpm/ml) (Takara, Ohtsu). Thereafter, the membranes were stripped using 0.1× SSPE, 0.1% SDS at 95°C, 10 min for rehybridization with the next probe. The membranes were also hybridized with human β-actin cDNA for standardization. The following probes were used for Northern blot analysis;

[1] To whom correspondence should be addressed.

cDNAs for Col IV (human α2-IV procollagen),[7] MMP-2 (human type IV collagenase-70 kD),[8] MMP-7 (human type IV collagenase-92 kD),[9] and TIMP-1.[10] Probes for MMP-2 and 9 were kindly supplied from Dr. K. Tryggvason. mRNA levels were quantitated by using a scanning laser densitometer (Densitron, Jookoo, Tokyo) and normalized according to the β-actin mRNA levels. Statistical comparisons, if necessary, were performed by Mann-Whitney's non-parametric t test.

Table I. Summary of 14 Cases with Pulmonary Squamous Cell Carcinoma

Case No.	Ope. date[a]	Age/Sex[b]	Histological typing[c]	Stage
1	6/4/92	67/F	moderate diff. SCC	IIIA
2	1/14/92	60/M	well diff. SCC	IV
3	11/26/91	54/F	moderate diff. SCC	IIIB
4	12/10/92	73/M	well diff. SCC	I
5	12/8/92	73/M	moderate diff. SCC	I
6	12/8/92	44/M	well diff. SCC	IIIB
7	9/18/92	69/M	well diff. SCC	II
8	4/14/92	65/M	poor diff. SCC	IIIB
9	2/25/92	62/M	poor diff. SCC	IIIA
10	9/11/92	61/M	moderate diff. SCC	IIIA
11	7/15/93	64/M	well diff. SCC	IIIB
12	8/26/93	60/M	well diff. SCC	II
13	5/13/93	72/M	well diff. SCC	I
14	3/15/93	58/M	moderate diff. SCC	I

a) Ope. date, operation date.
b) M, male; F, female.
c) diff. SCC, differentiated squamous cell carcinoma.

RESULTS

Col IV Col IV was linearly distributed in the interface between tumor cell nests and stroma, and in the walls of lymphatic and vascular vessels. At sites of Col IV disruption in the tumor stroma interface, marked lymphatic and vascular invasions were frequently encountered. Immunoreactivity was faint in such areas. Intensity of

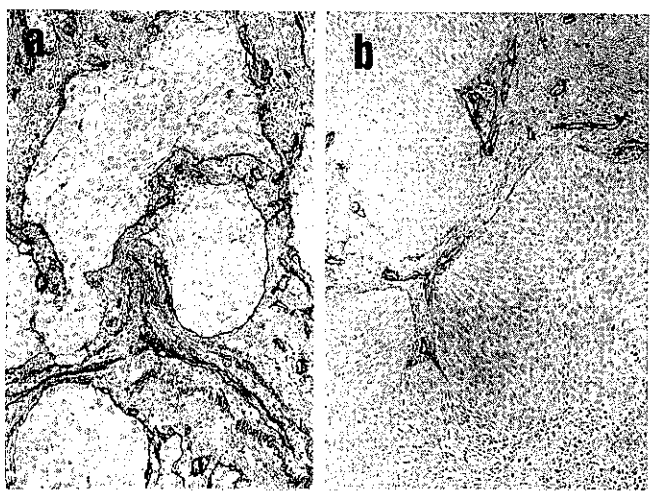

Fig. 1. Immunohistochemistry of type IV collagen. Type IV collagen is distributed linearly surrounding a nest of neoplastic squamous cells (a). Tumor nests of squamous cell carcinoma are surrounded by markedly disrupted type IV collagen (b). Note positive reaction of the stromal vascular walls. (×200)

Table II. Pathological Data and Results of Immunohistochemistry and Northern Blot Analysis

Case No.	TNM factor	ly, v factor	Col IV distribution	Immunohistochemistry		Northern blot analysis			
				MMP-2	TIMP-1	Col IV[a]	MMP[b]-2	MMP-9	TIMP-1[c]
1	T2N2M0	ly3, v1	limited BM[d]	++	++	0.41	0.84	0.36	0.76
2	T3N2M1	ly1, v1	moderate BM	++	+	0.42	0.38	0.09	0.38
3	T4N0M0	ly2, v3	limited BM	++	++	0.79	0.65	0.12	0.59
4	T2N0M0	ly1, v0	moderate BM	++	+	0.55	0.43	0.14	0.41
5	T1N0M0	ly0, v0	extensive BM	±	±	0.48	0.35	0.12	0.51
6	T4N2M0	ly1, v1	limited BM	++	+	0.54	0.42	0.11	0.56
7	T1N1M0	ly2, v3	limited BM	++	+	0.69	0.86	0.18	0.68
8	T3N3M0	ly3, v2	moderate BM	±	+	0.69	0.51	0.16	0.94
9	T3N2M0	ly2, v2	extensive BM	+	±	0.36	0.32	0.12	0.69
10	T3N1M0	ly1, v1	extensive BM	+	+	0.25	0.19	0.09	0.41
11	T4N2M0	ly2, v2	limited BM	++	+	0.39	0.58	0.18	0.68
12	T2N1M0	ly2, v3	moderate BM	++	+	0.31	0.85	0.25	0.12
13	T1N0M0	ly1, v0	limited BM	+	+	ND[e]	ND	ND	ND
14	T1N0M0	ly3, v3	moderate BM	+	+	ND	ND	ND	ND
P[f]						1.42	1.08	1.00	0.71
N[g]						0.15	0.20	0.17	0.65

a) Col IV, collagen IV. *b*) MMP, matrix metalloproteinase. *c*) TIMP-1, tissue inhibitor of metalloproteinase-1.
d) BM, basement membrane. *e*) ND, not done. *f*) P, placenta. *g*) N, normal lung tissue.

Col IV staining in the central part of the tumor was scored semiquantitatively based on the grading methods of ten Verde *et al.*[11] If positive immunoreactivity at the tumor-stroma border was found in more than 75% of the total circumference, then the case was scored as extensive basement membrane (extensive BM). If the positive length was between 25% and 75%, it was delineated as moderate BM, and if less than 25%, as limited BM (Fig. 1). By these criteria, 3 cases were extensive BM, 5 cases moderate BM, and 6 cases limited BM (Table II).

Clinically, 9 cases were found to have lymphnode metastasis. Col IV distribution had no apparent correlation with lymphnode metastasis, histological differentiation or clinical staging.

Results of Northern blot analysis of Col IV are shown in Fig. 2 and summarized in Table II. In contrast to the level of normal lung tissues, Col IV mRNA expression was enhanced in the tumor tissues. Comparison of the mRNA data with Col IV immunoreactivity disclosed paradoxically increased expression of Col IV mRNA levels in the limited BM group, although it was not statistically significant (Fig. 3).

MMP-2 and MMP-9 Positive immunoreactivity was observed in tumor cell cytoplasm, stromal fibroblasts, endothelial cells and bronchial epithelial cells in all the cases examined (Table II). MMP-2 was most intensely stained in the tumor cells, in particular, of marginal area of tumor nests (Fig. 4). In some parts of the tumor, however, MMP-2-positive tumor cells were also recognized without disruption of Col IV. The frequency of positive cells appeared to be high in the extensive BM group, followed by the moderate and limited BM groups, in that order.

Northern blot analysis demonstrated increased mRNA levels of MMP-2 as compared with that in normal lung

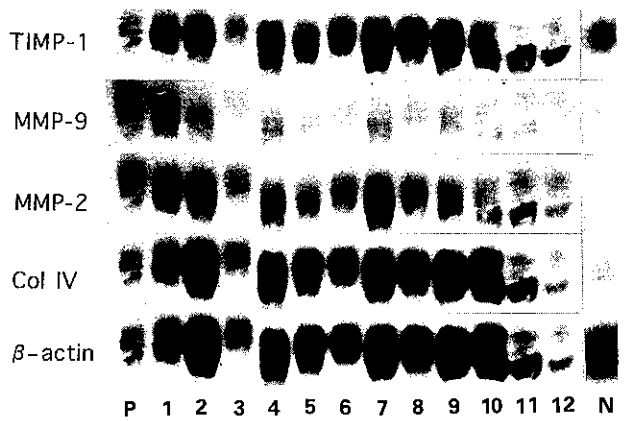

Fig. 2. Northern blot analysis of mRNA levels of type IV collagen (Col IV), matrix metalloproteinase(MMP)-2, MMP-9, tissue inhibitor of metalloproteinase(TIMP)-1 and β-actin in 12 cases of pulmonary squamous cell carcinoma. Elevated levels of MMP-2, MMP-9 and Col IV were detected in carcinoma tissues as compared with normal lung tissues (N). P stands for placental tissue.

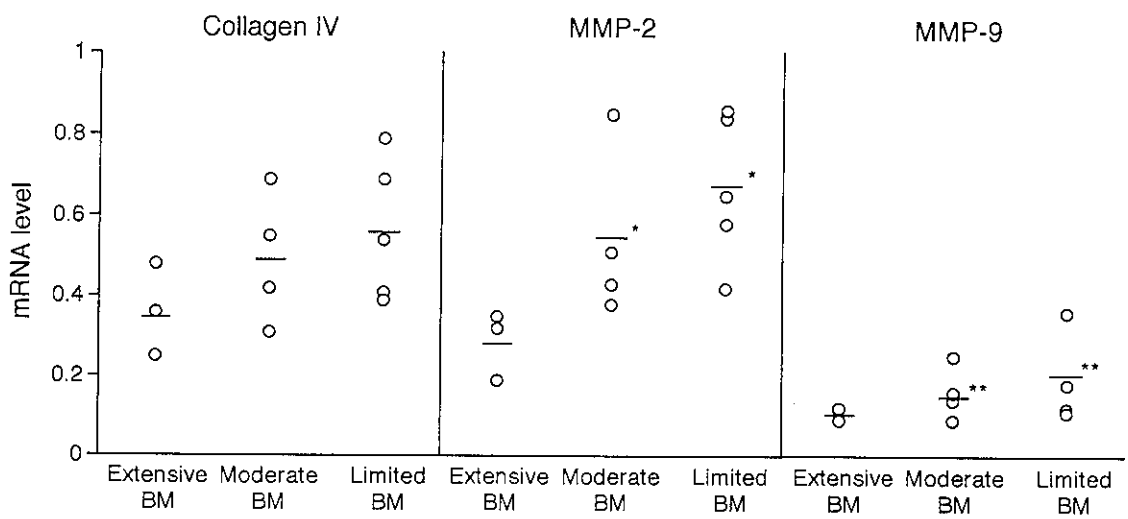

Fig. 3. Correlations between mRNA levels of collagen IV, matrix metalloproteinase (MMP)-2 and -9 and *in situ* disruption of basement membranes (BM) in lung squamous cell carcinoma. In a group of highly disrupted basement membranes (limited BM), expression of Col IV message levels was elevated as compared with those in the moderate and extensive BM groups (left). MMP-2 mRNA levels were significantly increased in the limited and moderate BM groups as compared with the extensive BM group ($*P<0.03$) (center). MMP-9 mRNA levels were also elevated in the moderate and limited BM groups as compared with the extensive BM group ($**$ $P<0.05$) (right). mRNA levels were normalized according to the β-actin mRNA levels.

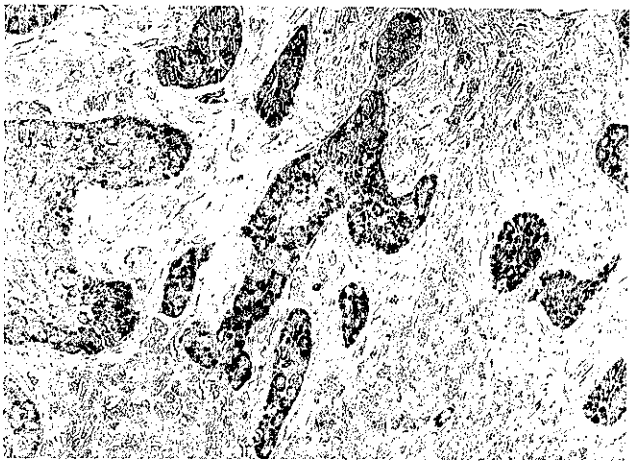

Fig. 4. Immunohistochemistry of matrix metalloproteinase (MMP)-2. MMP-2-positive cells are preferentially localized in the marginal area of the tumor cell nests. Stromal fibroblasts and endothelial cells are also positive. (×200)

tissues (Fig. 2). Expression of MMP-2 mRNA correlated with the results of *in situ* immunohistochemical detection of MMP-2 (Table II). Quantitative estimation of MMP-2 mRNA levels revealed a significant increase in the moderate and limited BM groups as compared with those in the extensive BM group (Fig. 3). The results of MMP-9 expression were similar to those of MMP-2, although elevation of MMP-9 mRNA levels was less conspicuous than that of MMP-2 (Fig. 3). There was, however, no significant correlation between mRNA levels of MMP-2 and MMP-9 and clinical staging or histological differentiation.

TIMP-1 TIMP-1 was observed in the cytoplasm of tumor cells, stromal fibroblasts, endothelial cells and macrophages in all the cases examined (Table II). Positive reactions of TIMP-1 were localized on the tumor cells, but were different from the pattern of MMP-2, not appearing preferentially in the marginal areas of the tumor nests.

TIMP-1 mRNA levels from tumor tissues were comparable to the level of normal lung tissues (Fig. 2). No significant correlation was detected in mRNA levels between TIMP-1 and Col IV. There was no relationship between TIMP-1 mRNA levels and clinical staging or histological differentiation.

DISCUSSION

The present study demonstrated enhanced expression of mRNA of Col IV and its degrading enzymes, MMP-2 and MMP-9, in primary pulmonary SCC. *In situ* immunohistochemical studies disclosed disruption of basement membranes where MMP-2-positive tumor cells penetrated into the stroma and vessel walls. The disruption and loss of basement membranes correlated with the elevated expression of MMP-2 and MMP-9 mRNA levels. Col IV mRNA levels were paradoxically upregulated in the tissues with less distinct basement membrane contour. It is therefore likely that the breakdown of basement membranes is due not to the depressed production of Col IV, but to the overproduction of MMP-2 and MMP-9 protein. Serum levels of MMP-2 protein were reported to correlate with clinical staging in patients with lung cancer with metastasis.[5]

There were focal areas of well-preserved Col IV reaction where concurrent strong positive reactions with MMP-2 were detected in the current immunohistochemical study. Campo *et al.*[2] obtained positive reactions of both Col IV and MMP-2 in tumor nests of ovarian cancers. In their studies, they used antibodies against both latent and active forms of MMP-2. It should be taken into account that the degradation process of Col IV may not be simply dependent on the MMP-2 levels; the chemical structures of the degrading enzymes may also be important for the invasion of tumor cells.

The cellular origin of degrading enzymes is a matter of controversy. Recent *in situ* hybridization studies on human colon cancer,[12, 13] breast cancer[14] and SCC of the skin[15] showed that MMP-2 mRNA expression was localized exclusively in the stromal fibroblasts, while strong immunoreactions were found in the tumor cells. This discrepancy can be accounted for by the presence of MMP-2 receptors on the cell surface of the tumor cells, which can bind free MMP-2 secreted from stromal fibroblasts.[14] Edonard *et al.*[16] recently demonstrated the cell surface receptors for MMP-2 in breast cancer cells *in vitro*. The preferential localization of immunoreactive MMP-2 in the marginal tumor cells of the tumor nests in the current study may suggest enhanced binding capacity of marginal tumor cells to MMP-2. Alternatively, trace message levels in comparison with protein content may exist with an extremely low turnover of MMP-2 synthesis in tumor cells. Future *in situ* hybridization studies will be necessary to solve this question.

TIMP-1 mRNA levels were not elevated in carcinomatous tissues from the present series, and were not correlated with mRNA levels of Col IV, MMP-2 and MMP-9. Recently, Tsuda *et al.*[17] found a high-molecular-weight type IV collagenase that was not inhibited by TIMP. Control of the balance between matrix collagen production and its degradation is complicated and may be regulated by unknown factors.

In this study, we could not obtain a significant correlation of *in situ* expression of Col IV, MMP, and TIMP proteins or their message levels with clinical staging and histological differentiation. The cases examined in the

present study may be too few, but lung SCC is well known to be heterogeneous.[18] Our sampling was only from a part of the tumor. The lack of correlation of the present data with clinical staging and histological differentiation may have been a consequence of the heterogeneous character of the lung SCC.

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